THE NEGATIVE RESULT OF AN ATTEMPT TO SENSITIZE WHITE MICE TO THE ACTION OF ULTRA-VIOLET LIGHT BY A DIET DEFICIENT IN TRYPTOPHANE.

by

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Harriette Chick (1), in a brief summary of the chief work which has been done on pellagra, points out that 50 per cent. of the protein of the maize endosperm is zein, a protein deficient in both tryptophane and lysine, and recalls the suggestions which have been made that pellagra is due to subsistence on a diet deficient in lysine and tryptophane.

She then reports that of three monkeys fed on diets whose protein content was low and especially poor in tryptophane and lysine, two developed mild skin lesions, while the third showed florid skin lesions and co-extensive localized oedema. In this third case, though addition of tryptophane to the diet was of little benefit, the addition of casein brought about a dramatic cure of the oedema and erythema.

It has been suggested that since aromatic amino acids absorb ultra-violet light rays (2), the tryptophane in the superficial layers of the skin may absorb the ultra-violet rays and prevent them from reaching the deeper layers of the skin. When the diet lacks tryptophane, it may be withdrawn from the superficial layers of the skin, leaving the deeper layers exposed to the toxic action of the ultra-violet rays. This would account for the affections of the exposed areas of the skin which occur in pellagra.

The object of the following experiment was to determine whether white mice could be sensitized to the action of ultra-violet light rays by feeding them on a diet deficient in tryptophane and, incidentally, in lysine. If such sensitization could be produced, it was intended to add tryptophane to the diet and see if it could be abolished.

The mice were kept in small earthenware dishes, one in each dish. They were fed once a day and weighed once a week. The hair was removed from a small

saddle-shaped area of the back, first with scissors and then with a moist mixture of barium sulphide and starch. The shaved mice were exposed to the rays of a mercury vapour quartz lamp by being put in a box directly underneath the lamp, and at a distance from it of something less than a foot.

The immediate object of the experiment was to find a time of exposure to the light such that the experimental mice would be burnt, but not the controls. During a preliminary experiment the control mice were fed on the diet always used in these laboratories. Rice cooked in milk and raw maize were given them daily, and hard-boiled eggs and lettuce were given them two or three times a week. The experimental mice were fed on maize alone. Sixteen mice, half experimental and half control, were exposed to the light for periods varying from two to five minutes. They were found to be equally sensitive to the light.

The experimental mice had been fed on maize alone for periods varying from six to twelve weeks.

In the main experiment the mice were fed on the following synthetic diets:

Casein (control) or Zein (experimental) -	20%
Sucrose - - - -	15%
Starch—arrowroot - - -	30%
Lactose - - - -	10%
Lard - - - -	7·5%
Clarified butter-fat - - -	7·5%
Salt mixture - - - -	5%
Agar Agar - - - -	5%

The above mixture was cooked in the form of a biscuit and crumbled. A small proportion of marmite or yeast was added before administering the diet to the animals.

Certain of the control mice were kept on the synthetic diet for eighteen weeks. The maximum weight attained by them was less than that attained by mice fed on rice, maize, and egg, so that at the end of the time they were rather undersized. Though their coats were less glossy than those of mice fed on a normal diet, they were apparently in good health.

It was found that the mice could not live much more than two weeks on the deficient diet. When, therefore, the experimental animals had been reduced to a moribund condition by subsistence on a synthetic diet lacking tryptophane and lysine, they were given rice and maize until they recovered. Then they were again given only the deficient synthetic diet.

At the same time a diet of rice and maize was substituted for the synthetic diet in the case of the control mice used for the light experiments, so that their treatment would be the same as that of the experimental animals.

About half-way through the time during which the mice were kept on the synthetic diets they were rayed. Thirty control and thirty-three experimental

animals, which had been on the synthetic diet for periods up to ten days at a stretch, were exposed to the light for periods varying from three to twenty minutes. On the dates of raying the ages of the mice varied between the limits of fifty and one hundred days, but mice of the same age were always compared together, and when possible from the same litter.

During the latter half of the period on the synthetic diets they were examined carefully every day for effects of the raying. On exposing the mice, both experimental and control, to the light, the blood vessels in the ears were observed to dilate, and afterwards the ears remained red and finally scarred. The skin on the backs of the different animals showed all degrees of brown pigmentation and desquamation, but in the average the experimental and control mice were found to be equally sensitive to the action of ultra-violet light. It was impossible to find a time of exposure which would burn the experimental but not the control mice.

SUMMARY.

Mice are not rendered more susceptible to the toxic effects of ultra-violet light upon the skin by omission of tryptophane and lysine from the diet.

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A STUDY OF HYDATID ANTIGEN.

by

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The nature of the antigen in diagnostic tests for hydatid infestation is still uncertain. In the complement fixation reaction the alcoholic extract of scolices described by Patterson and Williams (9), which contains a large amount of lipoid but only traces of protein, gives results which are specific and quantitatively similar to those obtained by the use of fresh hydatid fluid from infected sheep, which is distinctly richer in protein. In the present paper we record the results of quantitative precipitin tests carried out with these two antigens, and of some anaphylactic experiments. Incidentally, we have investigated the antigenic properties of some other preparations of scolices and of the scolices themselves.

Welsh, Chapman, and Storey (10), in this country, were the first to apply the precipitin reaction to the diagnosis of hydatid disease. As antigen, they used fresh human hydatid fluid. In their experiments, as in those of K. D. Fairley (4), who used carbolized hydatid fluid from infested sheep, no attempt was made to make the test quantitative. The addition of carbolic acid up to ·45 per cent. makes the antigen stable, prevents bacterial growth during the test, and allows the tubes to be read after thirty-six hours. In our experiments we have used carbolized hydatid fluid for this reason. Fairley found that if the serum to be tested were heated to 56°C. the reaction was abolished. In general, precipitins are not inactivated at so low a temperature, though irreversible inactivation takes place at temperatures of 70° and over. We were, therefore, unable to speed up precipitation by heating to 55° for one or two hours, but followed Fairley in carrying out the test at room temperature, reading the results after eighteen and thirty-six hours. This low temperature of inactivation seemed to us to be worthy of further investigation, since it presented a point of difference between the immunity of reactions to foreign protein of animal, vegetable, or bacterial origin, and those to helminthic protein, or lipoid-protein combinations.

We used for experiment a human serum, which in a dilution of 1 in 5 was capable of fixing 40 M.H.D. of complement, and put up a series of quantitative precipitin tests with this serum both unheated, and heated for half an hour at

40°, 45°, 50°, 56°, and 60°C. For each test a series of tubes containing equal parts (·5cc.) of varying dilutions of antigen and serum were put up, the antigen in dilutions of 1 in 10, 1 in 5, 1 in 2, and undiluted, tested against undiluted serum, and the serum in dilutions of 1 in 2, 1 in 5, 1 in 10, 1 in 20, and 1 in 40, against undiluted antigen. The following results were obtained after thirty-six hours.*

Antigen	1/10	1/5	1/2	P	P	P	P	P	P
Serum	P	P	P	P	1/2	1/5	1/10	1/20	1/40
Unheated	—	+	++	+++	++	++	++	+	—
40°C.	0	0	0	+++	++	++	++	+	—
45°	0	0	0	+++	++	++	++	+	—
50°C.	0	0	0	+++	++	++	+	—	—
56°	0	0	0	+++	++	+	—	—	—
60°	0	0	0	—	—	—	—	—	—
Control unheated	0	0	0	—	—	—	—	—	—
Control heated 56°	0	0	0	—	—	—	—	—	—

The precipitin begins to deteriorate at 50°, and is completely inactivated at 60°C. We were unable to re-activate it by the addition of fresh guinea pig serum as complement. Experiments carried out with less actively complement fixing sera yielded similar results.

The destruction of precipitin did not affect the complement fixing power of the serum, which in a dilution of 1 in 5 could still deviate 40 M.H.D. of complement. This fact seemed to point to the conversion of the precipitin into a precipitoid, which, as Kraus and von Pirquet (8) have demonstrated, takes place in the case of other precipitins when they are inactivated by heat. The addition of ·25 cc. of fresh unheated precipitating serum to the series of tubes in the above experiment containing this serum heated to 60°, and to those of the control negative serum heated for half an hour at 56°, gave the following results when read after a further eighteen hours at room temperature.

Antigen	P	P	P	P	P
Serum	P	1/2	1/5	1/10	1/20
Serum heated to 60°	—	+	++	++	++
Control heated to 56°	+++	+++	+++	+++	+++

The inactivated precipitating serum, though it had been unable to cause any precipitation with the antigen, inhibited its precipitation when active serum was added. No such inhibition is to be seen in the case of the control negative serum. Here is strong presumptive evidence of the presence in the heated serum of "precipitoid" unable to cause precipitation, but still capable of binding antigen

* P = undiluted serum or antigen. 0 = dilutions not tested. — = no reaction.

and deviating complement. Precipitin for hydatid protein appears to be similar to other precipitins, save in the readiness with which it is inactivated by heat.

The quantitative results of tests carried out upon some fifty different sera show also that while the reaction is specific, and there is no question here of the protein involved being derived from the host, i.e., the sheep, it is by no means so sensitive as the complement fixation test.

Closely allied to the question of the presence in a serum of substances capable of yielding a precipitin reaction is the capacity to confer passive sensitiveness. In our experience, this phenomenon happens somewhat irregularly, a finding which agrees with that of Bacigalupo and Grasso (1), who were only able to demonstrate passive sensitiveness with one of ten sera from hydatid infested patients. For this purpose we used virgin female guinea pigs of about 250 gms. weight, injected intraperitoneally with about 2 cc. of the serum to be tested, and using fresh hydatid fluid as an antigen, tested the sensitiveness of their plain muscle by the method of Dale and Laidlaw (2) after the lapse of forty-eight hours. In these tests it was necessary to first ascertain the maximal dose of antigen which could be employed without calling into play the stimulant effect of hydatid fluid upon plain muscle, which will be discussed later in the present paper.

The following experiment, in which a guinea pig of 230 gms. weight was tested forty-three hours after the injection of 2·5 cc. of the human serum referred to above, which was strongly precipitating and deviated 40 M.H.D. of complement, will serve to illustrate the low degree of sensitiveness which may be demonstrated even with a strongly precipitating serum.

	Dose of antigen.	Reaction of the plain muscle.
Right horn	4 cc.	Maximal, but slightly delayed.
Left horn	1 cc.	Nil.
	·05 mgm. histamine	Maximal.

In this case we were able to use as large a testing dose as 4 cc. in a bath of 120 cc. capacity, since in this concentration the sample of fluid used had no action on normal plain muscle. The weakness of the antigen probably explains the irregularity with which passive sensitiveness may be demonstrated.

In contrast to the constancy with which hydatid fluid yields precipitin reactions with positive human sera is the failure to elicit this reaction using as an antigen alcoholic extract of scolices evaporated to dryness and re-suspended in saline. The following protocol shows the sort of results obtained:

Antigen	1/20	1/10	1/5	1/2	P	P	P	P	P
Serum	P	P	P	P	P	1/2	1/5	1/10	1/20
Serum 1. Hydatid Fluid	—	—	+	++	++	+	—	—	—
Alcoholic extract	—	—	—	—	—	—	—	—	—
Serum 2. Hydatid Fluid	—	—	+	++	++	+	—	—	—
Alcoholic extract	—	—	—	—	—	—	—	—	—

These sera only deviated between 2 and 3 M.H.D. of complement with fresh hydatid fluid, while with the alcoholic extract they both deviated 4 or more M.H.D.

The alcoholic extract, though it contains traces of protein, does not appear to be able to act as a precipitating antigen, although as an antigen for complement fixation it is, if anything, more satisfactory than hydatid fluid. Of 129 positive sera tested by complement fixation, 44 gave identical results with both antigens, 45 gave more marked positive reactions with the alcoholic extract of scolices, 8 better positive reactions with hydatid fluid, while of the remainder, 17 gave positive results only with alcoholic extract and 15 only with hydatid fluid.

Several other workers have studied experimental hydatid anaphylaxis. Deluca (3), for example, sensitized guinea pigs with hydatid fluid and with extracts of cyst membrane. He found that the sensitizing power of hydatid fluid was extremely feeble, while that of extracts of membrane was more marked. Intravenous injection for the purposes of testing the sensitiveness of the animals fifteen to twenty days after the sensitizing injection gave the best results. In about 100 experiments he only observed three fatal shocks, and ten fairly severe ones with recovery. He was able to sensitize and test reciprocally with hydatid fluid and membrane extract, independently of the species of host from which the membrane or fluid was obtained. As regards hydatid fluid, this was toxic for normal guinea pigs in a dose of 8 to 10 cc., while 2 to 3 cc. was the order of dose necessary to produce shock in sensitive animals.

In our experiments, we sensitized virgin female guinea pigs with hydatid fluid, 1 or 2 cc. being used for a single sensitizing dose and 4 cc. when two sensitizing doses, each of 2 cc., were given at close intervals. We tested the acquirement of sensitiveness at various times after the sensitizing injection by the reaction of the isolated plain muscle of the uterus, suspended in oxygenated Ringer in a bath of 120 cc. capacity.

We were confronted with two difficulties in carrying out these experiments. There was, first, the possibility to be considered that sensitiveness demonstrated in this way might not be due to hydatid protein, but to the presence in hydatid fluid of serum protein from the host, i.e., the sheep. The second difficulty was presented by the pharmacological properties of hydatid fluid itself.

Giusti and Hug (6) studied the fluid from liver and lung cysts of sheep, and found that out of twenty-three batches of fresh fluid ten possessed pharmacological activity. They found that active samples produced contraction of the isolated plain muscle of the guinea pig in a concentration of 1 in 10,000 as great as that caused by 1 in 10,000,000 of histamine. Our first experiments were concerned with the confirmation of this observation.

Of ten separate batches of hydatid fluid from the liver and lung cysts of infected sheep, five were inactive in a concentration of 1 in 30, and five showed activity of varying degree in this concentration, i.e., in a dose of 4 cc. added to

the 120 cc. bath in which the plain muscle was suspended. In the majority of cases, when the fluid was active, its stimulant action resembled rather that of ergotoxin than that of histamine, in that a second dose added to the bath after changing the Ringer either failed altogether to cause a response or caused a much smaller one, which, in this latter case, finally exhausted the capacity of the uterus to respond to any further dose of hydatid fluid. Fig. 1 illustrates this type of effect, and shows that the plain muscle, though incapable of response to further doses of hydatid fluid, can still react to ergotoxin and to histamine. Fig. 2 shows that after the reaction of the plain muscle to ergotoxin has been exhausted it can still react actively to hydatid fluid.

This type of activity of the hydatid fluid did not appear to be related to bacterial infection. In one or two specimens, in which there was no guarantee of sterility, a histamine-like stimulant action on normal plain muscle was observed. The much more common ergotoxin-like effect made the use of active samples of fluid almost impossible for the determination of the presence of anaphylactic

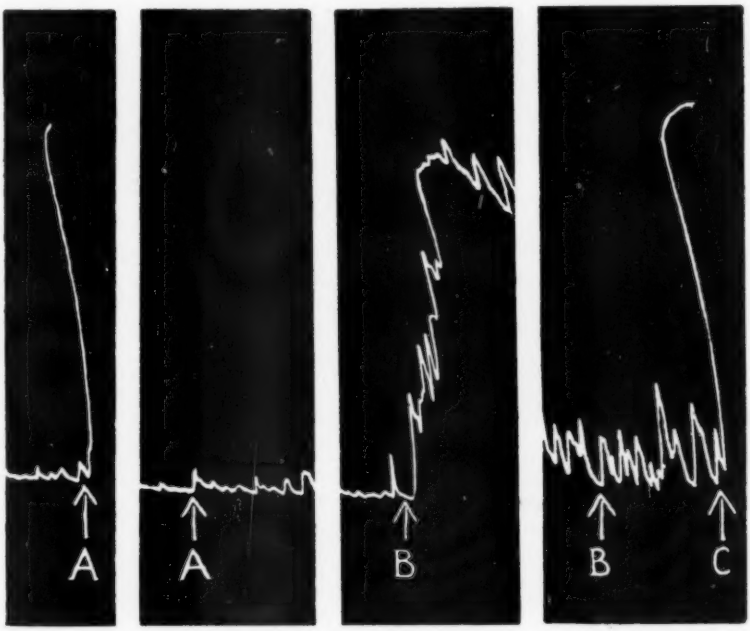

Fig. 1. Reactions of isolated uterus of normal guinea pig. At A, 4 cc. of hydatid fluid, at B .1 mgm. ergotoxin, and at C .01 mgm. of histamine added to the bath. Ringer changed after each response.

sensitiveness in plain muscle, since we had no guarantee that the anaphylactic uterus might not be sensitive to doses of the active principle, which were considerably smaller than those which were necessary to cause a response in normal smooth muscle, and if this were the case the response would resemble an

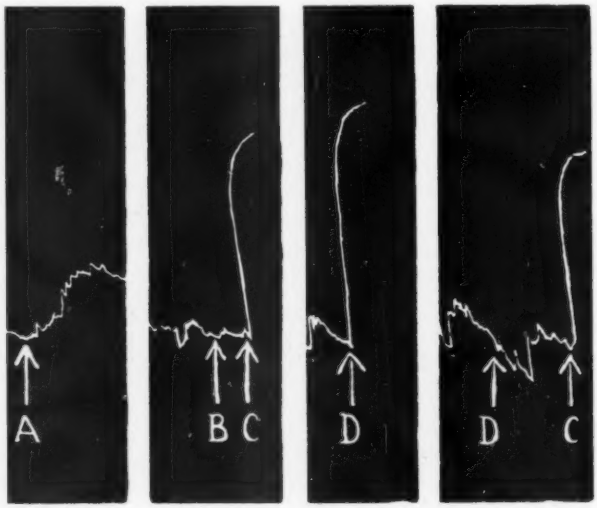

Fig. 2. Reactions of isolated uterus of normal guinea pig. At A, ·1, at B, ·2 mgm. ergotoxin, at C, ·01 mgm. of histamine, and at D, 4 cc. of hydatid fluid added to the bath.

anaphylactic one in the apparent desensitization which would follow the first stimulation. We have not observed any stimulant action of the fluid in the dilutions described by Giusti and Hug. It will be noted that in Fig. 1 the response to histamine, which is a maximal one, is caused by a concentration of 1 in 12,000,000, and that a concentration of 1 in 30 of hydatid fluid was used to produce a similar effect. Only once have we obtained a nearly maximal effect with a dose of 1 cc. This sample of fluid was sterile, but three days old. The other active samples at best gave half or quarter maximal reactions with a dose of 2 cc., a concentration of 1 in 60.

The resemblance of the action of this stimulant principle to that of ergotoxin is only a superficial one. A cat weighing 1·6 kilos had its spinal cord divided in the cervical region and its brain pithed under full ether anaesthesia. Altogether 45 cc. of hydatid fluid was injected intravenously in divided doses, each injection causing a small rise in blood pressure, distinctly more than that caused by an

equal quantity of saline. This treatment caused no reversal of the action of adrenalin, a phenomenon subsequently demonstrated on the same animal after the injection of 2 mgm. of ergotoxin.

Some samples of fluid were found to have a definite haemolytic effect on the sheep haemolytic system used for complement fixation, and we thought it possible that this might be co-related with the stimulant effect on the plain muscle. The stimulant principle was not seriously diminished in activity by heating to 55°C. for one hour, but was destroyed when the fluid was boiled for five minutes and filtered. The additional haemolysis which occurred in a haemolytic system on the addition of hydatid fluid was similar in amount whether fresh fluid or the same fluid heated at 55° for an hour was used. Fluid which had been boiled for five minutes and filtered caused no additional haemolysis, but showed instead a marked degree of anti-complementary power.

In regard to the other difficulty, we did not, as a routine, test all supposedly sensitive plain muscle first with sheep serum in order to exhaust any possible anaphylactic sensitiveness to sheep serum protein before testing the action of hydatid antigen. In the few experiments in which this was done we did not observe any anaphylaxis to sheep serum protein.

In the experiments in which hydatid fluid was used, both for sensitizing guinea pigs and subsequently testing their plain muscle, there was some irregularity in the degree of sensitiveness obtained.

The following table shows the results obtained in fourteen experiments. In most cases the reaction of one horn only has been given, but where two testing doses are given in the fourth column the lower is that with which the second horn was tested. It will be noted that in every case some variety of response was obtained. It is, however, open to question whether all of them were truly anaphylactic. The hydatid fluid used for testing was inactive to normal plain muscle in a dose of 4 cc. The size of the responses has been estimated by comparison with the effect produced by a dose of histamine varying from ·01 to ·02 mgm. The bath was of 120 cc. capacity.

No. of expt.	Wt. in gms.	Sensitizing dose.	No. of days sensitive.	Testing dose.	Reaction of the plain muscle.
1.	350	1 cc.	101	·5 cc.	Very small delayed.
2.	360	1 cc.	99	·25 cc.	¾ maximal response.
3.	335	4 cc.	33	2·0 cc.	Maximal (a second dose gave no response).
4.	280	4 cc.	22	2·0 cc.	½ maximal (the second horn gave repeated contractions to 3 cc. of hydatid fluid).
5.	260	4 cc.	28	2·0 cc.	¾ maximal.
				1·0 cc.	½ maximal.

No. of expt.	Wt. in gms.	Sensitizing dose.	No. of days sensitive.	Testing dose.	Reaction of the plain muscle.
6.	220	4 cc.	36	2·0 cc.	Maximal (a second dose gave no response).
7.	170	4 cc.	36	2·0 cc.	½ maximal (a second dose gave no response).
8.	174	4 cc.	36	2·0 cc.	¼ maximal response.
9.	200	4 cc.	36	2·0 cc.	Maximal (a second dose gave no response).
10.	235	4 cc.	20	2·0 cc.	Maximal (a second dose gave no response).
11.	204	4 cc.	21	2·0 cc.	Maximal, but repeated response to this dose.
12.	235	4 cc.	21	4·0 cc.	Maximal, but repeated doses gave smaller responses to this dose.
				1·0 cc.	Nil.
13.	214	4 cc.	64	2·0 cc.	Nil.
				4·0 cc.	½ maximal (a second dose gave no response).
14.	225	4 cc.	64	4·0 cc.	Maximal, but repeated doses gave a series of responses from ⅔ to a ½ maximal.

Of these fourteen experiments, in numbers 2, 3, 5, 6, 7, 9, 10, 12, and 13 the reaction observed was almost certainly anaphylactic, and in numbers 1, 4, 8, 11, and 14 the attainment of sensitiveness was doubtful. In nine out of fourteen experiments, therefore, we were able to produce anaphylactic sensitiveness with hydatid fluid. The chief reason for the irregularity of these results seems to be the small amount of protein in the fluid, and the inadvisability of using more than 4 cc. for any test. If sensitive plain muscle is more sensitive to the pharmacological action of fresh hydatid fluid, some of the nine cases which we have regarded as anaphylactic may instead be examples of this action.

The most interesting feature of these experiments was the presence in four cases of a peculiar type of response which we have never observed with sterile active fluid and normal plain muscle. A large reaction is obtained with the first dose of the antigen, but this does not exhaust the plain muscle, which goes on reacting repeatedly to subsequent doses of hydatid fluid, the Ringer in the bath being changed after each response.

In the cases in which the first response is not much greater than the subsequent ones, the reaction cannot be regarded as anaphylactic. In number four,

three successive contractions, each about ½ maximal, were obtained, but in the other three examples, numbers 11, 12, and 14, the first response was greater than the subsequent ones, and in the case of 12 this was so striking that we have regarded the first response as partly anaphylactic. It is not improbable that we might have found still other examples of this type of response had we invariably sought it by testing for desensitization after the first reaction to the antigen. Fig. 3, the record of number 14, is given as an example of this sort of response.

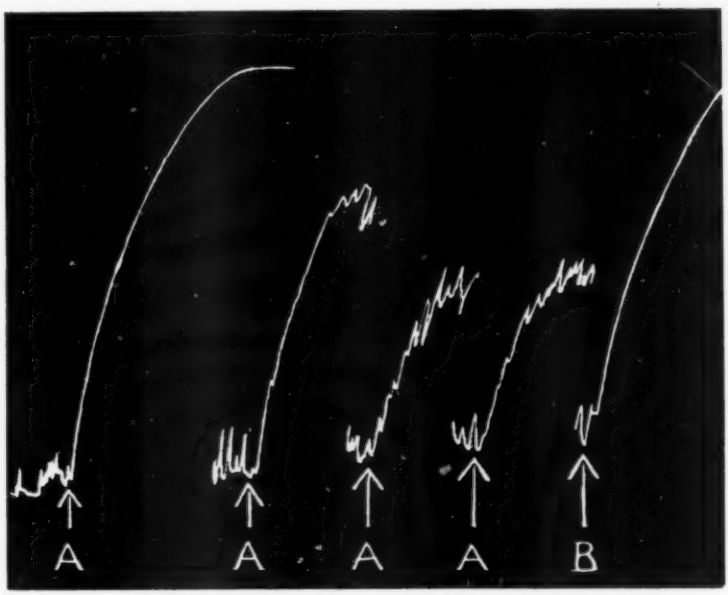

Fig. 3. Reaction of the isolated uterus of a guinea pig 64 days after a sensitizing injection of hydatid fluid. At A, 4 cc. of hydatid fluid and at B, .02 mgm. of histamine added to the bath.

With extremely sensitive anaphylactic plain muscle tested with doses which are not sufficiently large to invoke a maximal anaphylactic response, more than one reaction might be obtained, but the occurrence of three or four such responses with no very marked evidence of desensitization is clearly against the interpretation of the phenomenon as anaphylactic. In number 11, by the fourth addition of 2 cc. of hydatid fluid to the bath, we had almost succeeded in exhausting the activity of the plain muscle, the response having diminished from a maximal almost immediate one to a delayed ¼ maximal reaction. We have observed this

phenomenon on four other occasions: once when testing with saline suspension of alcoholic extract of scolices the uterine muscle of a guinea pig twenty-nine days after sensitization with hydatid fluid, once in an experiment in which alcoholic extract of scolices was used both for sensitization and testing, and twice in experiments in which a saline suspension of scolices was used for sensitization, and hydatid fluid for testing. In the first two cases there was no sign of any diminution in the response in a series of three successive doses, equivalent to ·5 cc. in one and 1 cc. of alcoholic extract in the other. The antigen in this dosage, when evaporated down to get rid of the alcohol and re-suspended in saline, has practically no activity as far as normal plain muscle is concerned. In the cases in which scolices were used for sensitization and hydatid fluid for testing, there was some evidence of exhaustion by the third response, as Fig. 4 shows.

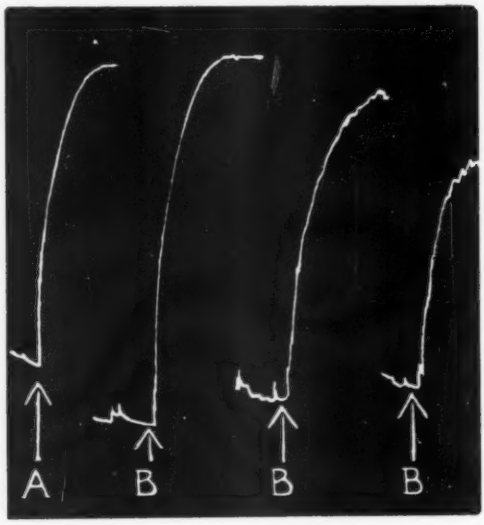

Fig. 4. Reactions of isolated uterus of a guinea pig 48 days after the subcutaneous injection of ·5 cc. of washed scolices. At A, ·02 mgm. of histamine, and at B, 1 cc. of hydatid fluid added to the bath.

The alcoholic extract of scolices was found to compare most unfavourably with hydatid fluid as an anaphylactic antigen, and despite its activity in deviating complement we have not found any certain evidence of its capacity in this direction, as the following experiments show:

Wt. in gms.	Sensitizing dose.	No. of days sensitive.	Testing dose. Alcoholic ext.	Reaction of plain muscle.
400	1 cc. hydatid fluid	104	·5 cc.	Doubtful response. The natural rhythm of this uterus was very marked.
330	,,	89	1 cc.	Nil.
350	,,	101	·5 cc.	Nearly maximal response to three repeated doses.
240	1 cc. alcoholic extract	28	1 cc.	Nil.
305	,,	22	·5 cc.	¼ maximal response.
300	,,	23	1 cc.	Nil.
240	,,	28	1 cc.	Repeated ¾ maximal reactions, not anaphylactic.

The difficulty in demonstrating any activity with this antigen in anaphylactic experiments is probably dependent on its small content of protein. In a patient who gave a well-marked immediate intradermal reaction following the injection of ·2 cc. of hydatid fluid, the intradermal injection of a similar dose of the suspension of evaporated alcoholic extract of scolices into the skin of the other arm caused about half as great a reaction. In ten minutes, with the hydatid fluid, the weal was as large as half-a-crown, while that due to the extract was of the size of a shilling. The sensitive human skin is an extremely delicate indicator, and it seems probable that the guinea pig uterus would be able to detect the antigen if it contained, say, twice as much protein, or if it could be used in large amounts. Unfortunately, we have not been able to obtain definite evidence of skin sensitiveness in either infected or sensitized animals.

A few experiments, in which ·5 cc. of thoroughly washed scolices were used to sensitize guinea pigs, showed clearly that hydatid protein in these was capable of acting as an anaphylactic antigen.

Wt. in gms.	No. of days sensitive.	Testing dose of hydatid fluid.	Reaction of plain muscle.
260	56	·5 cc.	Delayed ¾ maximal.
340	61	·25 cc.	Maximal.
		·1 cc.	Maximal.
310	61	·25 cc.	Nil.
360	63	·5 cc.	No definite response, very large rhythm of uterus.
200	42	1·0 cc.	Maximal.
190	42	·5 cc.	Very small response.

Wt. in gms.	No. of days sensitive.	Testing dose of hydatid fluid.	Reaction of plain muscle.
230	48	2·0 cc.	Maximal, no response to a second dose.
170	48	2·0 cc.	Maximal, no response to a second dose.
220	48	1·0 cc.	Maximal, repeated doses gave diminishing responses.
195	48	2·0 cc.	Maximal, repeated doses gave diminishing responses.

When these animals were killed we almost always found a mass of broken-down material, surrounded with dense fibrous tissue, at the site of the sensitizing injection in the abdominal wall. The absorption of antigen from these large injections must have been very small. In all but two of these experiments there was evidence of sensitization.

N. H. Fairley (5) pointed out that hydatid fluid and scolices seemed to be the only two available sources for antigen as far as complement fixation is concerned, and suggested that the antigenic factor was in some way related to the scolex. With this in mind, we have, with Miss Cowan, investigated the antigenic value of various preparations of scolices. We attempted first to obtain an antigen by tryptic digestion for varying periods, both of fresh scolices and of scolices which had been very thoroughly extracted with alcohol. The digests of extracted scolices showed no anti-complementary action, but were not as good for complement fixation as alcoholic extract.

Trypsin extracts of fresh solices, which were much more satisfactory, were made in the following manner:

1 cc. of fresh scolices, washed three times with normal saline, was placed in a sterile flask containing 40 cc. of ·4 per cent. sodium bicarbonate and ·1 gm. of Merck's trypsin. Five drops of chloroform were added, and the flask was then incubated at 37°C. At the end of 2, 4, 6, and 8 hours 10 cc. samples were drawn off, placed in sterile test tubes, and at once heated to 60°C. After cooling, they were neutralized with hydrochloric acid and placed in the ice chest. Before use for complement fixation the chloroform was driven off by bubbling sterile air through the liquid. These solutions proved to be potent antigens, even in a dilution of 1 in 20, and showed no tendency to react with syphilitic sera, but were weakly anti-complementary, even in high dilutions. Control solutions containing trypsin in the same dilution, incubated for the same time and treated in an identical manner, were devoid of any activity.

We also tried the effect of acid and alkaline hydrolysis with various strengths of acid and alkali. The acid hydrolysates had no antigenic value in complement fixation experiments, and in the few experiments which we made they appeared

to be incapable of acting as anaphylactic antigens. The alkaline hydrolysates, though of no value as anaphylactic antigens, were fairly potent in complement fixation tests, and showed but little tendency to deviate complement in the absence of specific sera.

The hydrolysis was carried out by heating 2 cc. of washed scolices with 10 cc. of normal NaOH under a reflux condenser on a water bath for from one to two hours. The mixture was then centrifuged, and the clear supernatant fluid neutralized with acid. At the neutral point a flocculent white precipitate appeared, which was separated by centrifugation.

The supernatant fluid and a suspension of the precipitate in normal saline were both antigenic. Three such experiments were carried out, and in none of them did these antigens show any marked anti-complementary action, even when undiluted. The results obtained with a number of sera by the use of one of these antigens were as follows: In this example, 4 cc. of scolices were heated with 10 cc. of normal NaOH for two hours, and a quarter of the precipitate obtained on neutralization suspended in 15 cc. of sterile saline constituted the alkaline hydrolysate tested.

The table shows the results of complement fixation tests by Harrison's method (7) upon eleven sera, using as antigens fresh hydatid fluid, alcoholic extract 1 in 10, and the alkaline hydrolysate in two dilutions, 1 in 5 and 1 in 10. The number of M.H.D. of complement in each of the four tubes comprising a test,

	REACTIONS TO VARIOUS ANTIGENS BY HARRISON'S METHOD															
	Fresh hydatid fluid				Alcoholic extract 1/10				Alkaline hydrolysate 1/5				Alkaline hydrolysate 1/10			
MHD Complement	2	3	4	2	3	4½	6	3	3	4½	6	3	3	4½	6	3
Number of serum																
1	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	−
2	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	−
3	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−
4	+	±	−	−	+	Tr	−	−	+	+	+	−	+	+	±	−
5	+	+	±	−	−	−	−	−	+	+	+	−	+	+	+	−
6	+	+	±	−	+	+	+	−	+	+	+	−	+	+	+	−
7	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	−
8	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	−
9	−	−	−	−	+	±	−	−	+	+	+	−	+	+	±	−
10 Strongly positive Wasserman	−	−	−	−	−	−	−	−	+	±	−	−	+	Tr	−	−
11 Normal Control	−	−	−	−	−	−	−	−	+	±	−	−	+	Tr	−	−

the fourth being a control containing serum but not antigen, is placed at the top of each column. An additional control for the whole series was provided by an extra series of four tubes for each antigen, containing sensitized red cells, the first containing, in addition, complement but not antigen, and the others, antigen with varying amounts of complement. This series of controls is not shown in the table, as all the tubes were negative.

A reading of + + + − indicates that haemolysis was completely inhibited in the first three tubes, and was not inhibited in the control. The sign ± indicates partial inhibition, and "tr" that a trace of inhibition has occurred.

It seems likely that this antigen may prove to be useful, though at present it is in the experimental stage. The tendency to give pseudo-positive reactions with non-specific sera will in all probability be avoidable by using the antigen in higher dilutions.

Acetone extract of scolices does not appear to be as serviceable an antigen for complement fixation as the alcoholic extract, which for this purpose is the best antigen available. We have been unsuccessful in the endeavour to procure an antigen which can be kept in a dry state and handled quantitatively for all types of immunological reactions.

CONCLUSIONS.

1. Hydatid fluid can be used as an antigen in all the diagnostic immunological tests, and can function as an anaphylactic antigen.

2. A quantitative precipitin test is described. The specific precipitin in the serum of infested human patients resembles other precipitating sera in being inactivated by heat with the formation of a precipitoid, and differs from them only in the low temperature of inactivation.

3. Alcoholic extraction of scolices is only useful as an antigen for complement fixation. It acts neither as a precipitating nor as an anaphylactic antigen.

4. Hydatid fluid contains a substance with a stimulant ergotoxin-like action on plain muscle.

5. An irregular reaction of sensitized plain muscle to various antigens is discussed.

6. The antigenic value of various preparations of scolices has been investigated.

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A COLOUR TEST FOR GUANIDINE BASES, TOGETHER WITH SOME PHYSIOLOGICAL APPLICATIONS

by

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(Submitted for publication 12th August, 1924.)

The factor which has hitherto made the investigation of Guanidine bases so difficult a matter has been the absence of any certain method of identifying and estimating them when present in small quantity.

The method of W. Koch (5), employed also by Noel Paton and his collaborators in their work on tetany (7), consists in precipitating the organic bases from solution by hot sodium acetate and mercuric chloride; albuminous bodies having previously been removed by tannin. From this precipitate methylguanidine is finally obtained as a gold salt, after various other bases and salts have been removed. Now it has been shown by Baumann and Ingvaldsen (1), and by Greenwald (3), that creatine can be oxidized to methylguanidine by mercuric acetate under the conditions employed by Koch. The method is therefore inapplicable to the detection of methylguanidine in muscle or in urine (following on parathyroidectomy) when creatine is known to be excreted. Moreover, it is quite inapplicable to the detection of minute traces of methylguanidine, since its precipitation by mercuric acetate is only very incomplete (3).

For this method Greenwald has substituted a very long one (3), from which he concludes that methylguanidine is absent from meat. A certain amount of doubt attaches to his method, however, since he subsequently found it to be inapplicable to the recovery of the base from urine into which it had been placed (4).

Under these circumstances, the following colour reaction, which I accidentally discovered during an attempt to obtain methylguanidine from muscular tissue, may be appreciated.

METHODS.

In the well-known Weyl test for creatinine, a freshly prepared weak solution of sodium nitroprusside is added to the solution to be tested; the

solution, on being made faintly alkaline, turns a ruby-red colour if creatinine is present. If, however, the nitroprusside solution has been exposed to air and light, and has been allowed to stand for about a day, its colour changing from brown to blue and then to a dirty brown, it is able to produce a red colouration, not only with creatinine, but also with creatine, with guanidine and with its methyl derivatives.

In order to prepare the reagent, a moderately strong solution of sodium nitroprusside is exposed in a shallow layer to the sunlight for a day or two. It may then be kept in a bottle indefinitely, and does not deteriorate. In order to detect the various guanidine bases, one of the following two procedures is adopted:

(1) The solution to be tested is made alkaline ($\frac{N}{30}$ is a suitable alkalinity to work at). To about 10 cc. of such a solution about 5 drops of the reagent are added. If a red or red-brown colouration is produced, creatine, creatinine, or one of the free guanidines is present. Guanidine may at once be recognized by the fact that it gives the test in neutral solution; creatinine may be distinguished from the other bases by the fact that, if the solution which is being tested is allowed to stand for about two hours, then at $\frac{N}{30}$ alkalinity the red colouration disappears, turning to a dirty olive green. Any remaining red colour is due to methyl- or dimethyl-guanidine, or to creatine (guanidine having been previously eliminated). This method is, therefore, incapable of differentiating between methylguanidine, dimethylguanidine, and creatine. If, however, the creatine has been previously converted into creatinine by heating it in a slightly acid solution in an autoclave for about half an hour, then any red colour that persists indicates the presence of methylguanidine or dimethyl-guanidine, guanidine having been previously excluded.

The test is very sensitive, and the colour may still be recognized at a dilution of 1 to 400,000. In carrying out the test it will be found that the red colouration passes through a period of maximum intensity and gradually fades again, remaining at a constant intensity after about two hours. By examining the solution colorimetrically after this fading has occurred, a quantitative estimation of guanidine bases can be made.

The specificity of the test may be judged from the fact that it cannot be obtained with liver extract.

(2) The following method, which is designed to detect guanidine bases directly in the presence of creatine and creatinine, may also be employed. It is not, however, as sensitive as that above described.

If to the "nitroprusside" reagent there is added, just before use, about double its volume of strong ammonia, then the colouration which it gives with creatinine is only transitory, fading entirely after slight warming. Creatine

likewise does not give the test. The colouration due to the guanidine bases persists.

APPLICATIONS.

(1) *Absence of free guanidine bases from Muscle.*

When the ammoniacal reagent is added to a weakly alkaline extract of meat no colouration is produced. A control experiment, to which methylguanidine has been added, gives the colouration.

If the test is carried out by the first method, then an alcoholic extract of the muscle is first made; the alcohol is then evaporated off and the filtered extract made aqueous. Proteins, which might disintegrate and yield guanidine during the subsequent autoclaving with acid, are thus removed. When the creatine in this extract is now converted into its anhydride, and the solution, on being made faintly alkaline is treated with "nitroprusside" reagent, the red colouration that is produced fades to a green, and no red colour remains. Guanidine bases are therefore absent.

The ease with which methylguanidine can be obtained from creatine by oxidation may account for its isolation from muscle tissue by various workers. Smorodinzew, for example (8), has obtained ·08 per cent. methylguanidine from the muscles of the horse. His method involved the use of silver nitrate and of dilute nitric acid, substances which should both bring about the oxidation of creatine into methylguanidine; no reliance is therefore to be placed on his results.

Similarly, Brieger (2), in his well-known work on the ptomains of putrid meat, obtained a considerable amount of methylguanidine. By applying the "ammoniacal nitroprusside" colour reaction, above described, to aqueous extract of beef, which had been allowed to putrefy, I have failed to obtain any colouration. Greenwald (3) was similarly unable to obtain methylguanidine from beef that had been standing for several days.

(2) *Urine.*

To 10 cc. of a fairly colourless urine five drops of the reagent are added. Strong caustic soda is then added drop by drop till the urine just turns bright red (creatinine). If this urine is now allowed to stand for about two hours the colouration almost entirely disappears. The faint colouration that remains can be detected even after several days' standing.

As traces of creatine might possibly be suspected in the urine, I have tried the effect of previously autoclaving it for about half an hour with weak acid at 120°C.; even then the colouration persists. It must, therefore, be concluded that human urine contains traces of free guanidine bases.

(3) *The time of appearance of creatine during embryonic development of the chick.*

According to E. Mellanby (6) creatine does not occur in the chick embryo till the twelfth day. It is at this time also that the liver begins to form, and, according to Mellanby, the rate of growth of the liver is roughly proportional to the rate of creatine production during embryonic development. He draws the conclusion from this that the formation of creatine in vertebrates (it is absent in invertebrates) is connected ontogenetically and phylogenetically with the formation of a liver.

By removing the heart of a chick embryo at the fourth day of incubation, and boiling it in a cubic centimetre of water for about ten minutes, so as to dissolve any creatine which might be present, I have had no difficulty, on applying the "nitroprusside" test, in detecting the colouration characteristic of creatine and the guanidine bases.

Four embryos were removed from eggs on the fifth day of incubation. The developing heart was dissected out from each and placed in 10 cc. saturated picric acid. The rest of the embryo was collected into another flask of picric acid. The material was then autoclaved for half an hour at 120°C., and Jaffe's test applied. In both the developing heart and the developing body tissue creatine could be demonstrated very clearly.

It is seen, therefore, that creatine is present both in embryonic cardiac muscle and in developing skeletal muscle long before the liver develops.

It may, therefore, be concluded that, whatever the effect of the liver on creatine metabolism in adult life may be, there is no evidence to show that creatine formation in the embryo is connected with the development of a liver.

SUMMARY.

1. A colour reaction for creatine and for the guanidine bases is described. By this means it is shown that:

(a) Methylguanidine is not present in muscle tissue, nor in putrid meat extract.

(b) Free guanidine bases are present in minute amount in human urine.

(c) Creatine occurs in developing skeletal and cardiac muscle of the chick embryo on the fifth and probably even on the fourth day, i.e., long before the liver develops (twelfth day). There is, therefore, no relation between creatine formation and liver development.

I desire, in conclusion, to express my thanks to Professor T. Brailsford Robertson, in whose laboratory this work was carried out, for the help and advice he has given me, and for procuring the guanidine bases used in this investigation.

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A COLORIMETRIC METHOD FOR THE ESTIMATION OF GUANIDINE AND METHYLGUANIDINE.

by

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The doubt which exists pertaining to the metabolism of guanidine and methylguanidine may almost certainly be ascribed to the lack of a suitable method for the estimation of these substances. Whereas small quantities of the guanidine derivatives, creatine and creatinine, may be estimated with speed and accuracy (4), the methods for the estimation of guanidine and methylguanidine are lengthy and cumbersome, taking several days to perform, and yielding results of questionable accuracy. Thus those methods in which Engeland's (3) mixture of mercuric chloride and acetic acid is used as a precipitating reagent at one stage, fail to give a correct estimate of guanidine or methylguanidine in biological materials, owing to the fact that any creatine present is partly oxidized by this reagent to methylguanidine (5). The usefulness of Koch's method (6, 7), and its modifications (1) is greatly diminished by this fact. Similarly, the methods using silver nitrate and barium hydroxide as a precipitant are rendered invalid by the fact that the silver oxide produced will oxidize the creatine to methylguanidine (2).

The investigations from which the following method was developed were initiated by an observation made by O. W. Tiegs,* that a solution of sodium nitroprusside, which has stood for some time in contact with air and light, will give a colouration with methylguanidine, as well as its derivatives, creatine and creatinine. The reagent so produced is a dark-brown opaque solution, which on warming in a slightly alkaline solution will give a reddish colouration with methylguanidine, creatine, and creatinine. Tiegs finds that the colour given by creatinine fades on standing in strongly alkaline solutions, while that given by methylguanidine does not.

The author undertook this work with an idea of determining the substance present in the decomposed nitroprusside that was responsible for the colour

* Described in the paper preceding this.

production, and, further, of investigating the possibility of instituting a condition which would inhibit the production of colour by creatine and creatinine. The method and results are outlined below.

Method.

The reagent employed consists of:

$\frac{M}{5}$ Sodium nitroprusside

Na_2 Fe $(CN)_5NO.2H_2O$ 59·6 gms. per litre.....100 ccs.

$\frac{M}{5}$ Potassium ferrocyanide

K_4 Fe $(CN)_6$ $.3H_2O$ 84·4 gms. per litre.....100 ccs.

5 N Sodium hydroxide 100 gms. per litre..... 50 ccs.

The materials mixed in the above proportions will keep indefinitely. About fifteen minutes before the reagent is used, an equal bulk of

Hydrogen peroxide (H_2O_2 10 vol.)

is added. A light yellowish-green reagent is thus produced, which will only keep an hour or so. It is necessary to make the required dilution with the peroxide for every set of estimations.

In a dilution of 1 in 20 this reagent will give an intense red colour with guanidine and methylguanidine, no colour being produced by either creatine or creatinine. The colour obtained is directly proportional to the guanidine content, and is estimated by comparison with the colour which is produced under similar conditions by a known amount of guanidine. The standard for comparison is made up from guanidine carbonate which has been dried in an evacuated desiccator over $CaCl_2$.

Standard guanidine carbonate : 10 cc. contains 10 mgms. of guanidine is a convenient strength. It is made by dissolving ·1524 grms. of the dried carbonate in 100 ccs. of H_2O. If preservatized with a few drops of toluol the solution will keep without change for some weeks. Guanidine carbonate is procurable on the market in a high degree of purity. For reasons which will become evident, the author has used in these investigations molecularly equivalent solutions of guanidine and methylguanidine. However, the calculation of the estimation is greatly facilitated by using a standard containing a suitable multiple of 1 mgm. of guanidine for comparison with solutions of unknown strengths.

It is necessary to standardize the methylguanidine by its nitrogen content, as the fact that it is very deliquescent and combines with ease with the carbon dioxide in the air renders it impracticable to standardize it by weight.

Procedure.

The amount of solution in which the guanidine or methylguanidine is to be estimated is chosen so that it contains approximately 5 mgms. It is then diluted to 90 ccs. with distilled water, and 5 ccs. of the prepared reagent added. The volume is then made up to the 100 cc. mark. The colour produced is determined by comparison with a standard prepared under identical conditions and containing exactly 5 mgms. of guanidine, i.e., 5 cc. of standard guanidine solution. The quantity of guanidine is directly proportional to the amount of colour produced. The production of colour takes some time to fully develop, but will reach its maximum intensity in about an hour at room temperature. It would develop more quickly if warmed, but it is inadvisable to heat above 50°C.

The colour ratio between the standard and unknown solutions may be read with any suitable colorimeter. A Bock-Benedict instrument with a source of light from a frosted screen illuminated by a 400 c.p. "daylight light" was used in the following tests. The amount of unknown solution that is taken is controlled by the concentration of the guanidine or methylguanidine that is expected. Thus solutions of very low concentration necessitate the direct addition of the reagent. In such cases the reagent is added in the proportion of 1 cc. to 20 cc. of the unknown solution, and the standard solution for colorimetric comparison prepared under identical conditions to contain a known amount of guanidine of the same order as that of the unknown. That the guanidine or methylguanidine content of solutions containing 1 part in 10,000 may be estimated with a high degree of accuracy is shown in the following experiment:

Estimation of small quantities of Guanidine or Methylguanidine.

Two cc. of $\frac{M}{50}$ guanidine was diluted with 100 cc. of distilled water, giving a solution containing 1·18 milligrams per 100 cc. or 1·18 parts in 10,000. Twenty cc. of the guanidine solution was taken and 1 cc. of reagent was added. The colour produced was compared with that produced under similar conditions by 20 cc. of a solution containing 5·9 milligrams of guanidine per 100 cc. (5 cc. of $\frac{M}{50}$ in 100 cc. H_2O). The results are tabulated below:

Solution.	Colorimeter reading after one hour.	Colour ratio.	Guanidine content in Milligrams per 100 cc. Calculated.	Observed.
Standard	6·1	—	5·9	—
Unknown	15·1	$\frac{6·1}{15·1}$	2·36	2·38

The error of about 1 per cent. is within the limit of error exhibited by the colorimeter. An average of six readings was taken. The variability of ·2 mm. was about the average under the conditions of the experiments.

That methylguanidine produces a colour of similar depth to its equivalent molecular proportion of guanidine is shown in the following table. The dimethyl derivative used was of doubtful purity. The methylguanidine was standardized by its nitrogen content (Folin's micro method). The readings after two hours are tabulated:

Substance.	Colour ratio produced by 1 cc. of $\frac{M}{5}$ in 100 cc. Standard Guanidine.	Derivatives.	Colour ratio within ± 1 %.
Guanidine	15·0	—	—
Methylguanidine	—	15·2	1·00
*Dimethyl guanidine	—	22·5	·69
Creatine	—	Nil	0
Creatinine	—	Nil	0

Methylguanidine in a mixture with guanidine may be estimated by its content of guanidine nucleus. An error of 20 per cent. (15 in 72) depending on the extra CH_3 group in the gross weight of the methyl derivative is instituted. However, if methylguanidine only is present the amount may be estimated as accurately as guanidine, i.e., to within 1 per cent. of the amount present. It is convenient to state the methylguanidine and guanidine content of a mixture in terms of guanidine.

The estimation of the amount of guanidine and methylguanidine present in such mixtures and mixtures of guanidine with creatine and creatinine are given in the following protocol:

Volume = 100 cc. containing:	Colour reading after 1 hour at 30° C.	Cc. of $\frac{M}{20}$ guanidine present in 100 cc. Calculated.	Observed.
Guanidine 1 cc. $\frac{M}{20}$ Standard	15·1	1·0	1·0
Creatine 50 mgms. Creatinine 50 mgms. Guanidine 1 cc. $\frac{M}{20}$	15·1	1·0	1·0
Methylguanidine 2·5 cc. $\frac{M}{40}$	12·4	1·25	1·21
Methylguanidine 4·0 cc. $\frac{M}{40}$	7·6	2·0	1·98
3 cc. $\frac{M}{40}$ Guanidine	10·1	1·5	1·48

* As the dimethyl derivative was probably very impure, the ratio cited for this substance should not be accepted until it is satisfactorily confirmed.

The above results show that correct estimates of guanidine and methylguanidine may be made with an accuracy of ± 1 per cent., even when they are in the presence of relatively large quantities of creatine and creatinine.

Creatine is often highly contaminated with free methylguanidine. For this reason it is necessary to purify the creatine before it is possible to demonstrate its inability to produce a colour with the guanidine reagent. The following procedure is highly satisfactory. The creatine (5 gms.) is dissolved in about 40 cc. of H_2O by warming the solution, a few drops of ammonia are added, and then twice the volume of absolute alcohol. On standing in a cool place the creatine separates in shining crystals within a few hours, and may be filtered off and washed with a little alcohol. The methylguanidine impurity is set free from its salts by the ammonia, and remains in solution in the alcohol. The procedure is repeated, if necessary. About 80 per cent. of the original creatine is recoverable after two such crystallizations.

The application of the method to urine and tissues will be described by the author in a later paper.

I am indebted to Mr. L. A. Ray for a sample of creatinine zinc sulphate, from which the creatinine used in this work was separated.

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THE NATURE OF THE FACTORS WHICH DETERMINE THE DURATION OF THE PERIOD OF LAG IN CULTURES OF INFUSORIA

by

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INTRODUCTION.

Our knowledge of the circumstances which determine the permeability of the external surface-layer of cells has been greatly augmented in recent years. We are now in a position to formulate certain general rules concerning the penetration of cells by chemical substances, rules which may not be universally applicable, it is true, but which nevertheless afford an important guide to anticipation and some measure of insight into the probable nature and spatial arrangement of the several components of the surface-layer.

Since fat-soluble substances enter the cell, as a rule, with comparative ease, while water-soluble substances which are not soluble in fats, with certain definite exceptions, enter the cells also, but with comparative difficulty (3), it appears probable that the surface-layer of the cell consists of an emulsion of fatty substances in a protein menstruum (9), which is of such a nature that a section resembles a mosaic, in which the larger proportion of the area is lipoidal and the smaller proportion, constituting the interstices between the lipoid globules, is a semi-solid solution of protein which is more concentrated than the underlying cell fluids (14). Any circumstance which widens these interstices, such as coalescence of the fat-droplets, leading to the formation of larger masses separated by wider interstices, must therefore enhance the permeability of the surface for water-soluble substances (16). Hence fat-solvents, such as ether or chloroform, and substances which reduce surface-tension, such as bile-salts and saponin, act as cytolytic agents by permitting the entrance of excess of water into the cell (11).

The conditions which determine the permeability of the nuclear membrane, however, are much less clearly comprehended. Little is known of the properties of this surface, which, nevertheless, must be of supreme importance in the life of the cell. When, indeed, we consider the fact that the whole course of develop-

ment is demonstrably controlled by the chromosomes contained within the nuclei, it is clear that the partitioning-off of the nuclear constituents during inter-divisional periods cannot be devoid of significance. We have, here, a membrane within a membrane; one surface with special properties of penetrability which is totally enveloped within another surface, also possessed of special, and, as we shall see, different qualities of penetrability. Any substance which is to gain access to the nucleus during the inter-divisional period, therefore, must pass two selective barriers. Two different assortments are imposed upon substances entering the nucleus, and we might anticipate that the substances falling within both assortments, and simultaneously satisfying the two different sets of conditions which are prerequisite to penetration, must be comparatively few in number and limited in variety.

In correspondence with this anticipation, we find that many substances which are familiar components of cytoplasm are absent from the nucleus. Chlorides, phosphates, and potassium are among the constituents of cytoplasm which are lacking in the nucleus (7). On the other hand, it has been suggested that the nucleus may contain greater abundance of free amino-acids than cytoplasm (1). The evidence in support of this supposition is not yet conclusive, but even if we admit that it is possibly true, we are still without a clue to the nature of the amino-acids which succeed in penetrating the nucleus, whether, that is, all amino-acids gain access thereto or only a limited number of them.

An experiment which very clearly illustrates the differing permeabilities of the exterior surface of the cell and the nuclear membrane was published by the author some years ago (13). If infusoria *(Paramoecium)* are immersed for a few minutes in a solution of methyl green, carefully purified from methyl violet, and are then rapidly washed by centrifugalization, the infusoria for one or two minutes will appear faintly stained by the dye which later diffuses out of them. The nucleus, however, is not stained. If, at this stage, a drop of a very dilute solution of a salt of some heavy metal (e.g., copper) be introduced, the infusoria are instantly killed and, almost at once, the cytoplasm becomes colourless, while the meganucleus is deeply stained. Either the heavy metal salt itself or the train of circumstances arising out of the death of the organism renders the nuclear membrane permeable by the dye, which, since it forms an insoluble compound with nucleic acid (8), leaves the cytoplasm, in which it appears to be merely in solution, and becomes concentrated in the nucleus. But in the living organism, although it can freely penetrate the cytoplasm, it is unable to gain access to the nucleus.

At the moment of nuclear division the nuclear membrane is dissolved, and the barrier hitherto interposed to limit the exchange of substances between nucleus and cytoplasm is removed. Without a doubt this fact must be one of fundamental significance in the mechanisms of reproduction and development.

It becomes, therefore, a matter of considerable importance to ascertain the nature of the conditions which govern the penetration of chemical substances into the nucleus and the issuance of others out of the nucleus. Macallum has, indeed, suggested that chromatin may possibly issue from the nucleus, and that the nuclear membranes of different types of cells may differ in the type of chromatin to which they are permeable (7). Even if this be the case, however, we still remain ignorant of the nature of factors which confer upon the nuclear membrane these peculiar properties, and also of any means by which its permeability may be modified.

In essaying to ascertain the nature of the substances which may influence the permeability of the nuclear membrane, we are restricted to the study of those substances which are also able to penetrate the exterior surface of the cell, and the field of inquiry is therefore limited in a number of directions. The most serious obstacle to investigation of this question, however, has hitherto consisted in the lack of any criterion of alteration of the permeability of the nucleus. Altered permeability of the exterior surface of the cell is indicated by loss or absorption of water, which may be ascertained and measured in a variety of ways, or by the passage of electrolytes through the surface-layer, which may be detected by changes of electrical conductivity. But such signs are lacking, or, at any rate, have not hitherto been detected in the nucleus.

Lillie (6) has proposed to utilize for this purpose the formation of indophenol, by the oxidation of a mixture of α-naphthol and paraphenylenediamine, as an indicator of enhanced permeability of the nuclear membrane. He has ascertained that the formation of indophenol takes place first at the surface of the nucleus, and later at other protoplasmic surfaces within the cell (5). These observations have been confirmed and extended to vegetable cells by Osterhout (10). Lillie has furthermore shown that the passage of an alternating current of considerable intensity through the leucocytes in frog's blood greatly accelerates the oxidation of indophenol at the periphery of the nucleus. This he refers to an increase of the permeability of the nuclear membrane due to the current, thus leading to more rapid liberation of oxidizing enzymes from the nucleus.

Unfortunately this method is not one of general applicability. The reagents employed are by no means innocuous. In fact, they are so toxic for infusoria *(Enchelys)* that I have found it necessary to dilute them to one-fortieth of the concentration employed by Lillie (that is, a mixture of a saturated aqueous solution of α-naphthol with an equal volume of a 1 per cent. aqueous solution of paraphenylenediamine). But at this low concentration indophenol, if formed, is not distinguishable. This test is, therefore, limited in its applicability to those cells and tissues which chance to be especially resistant to the toxic effects of the reagents.

The growth of animals and plants and, in general, the multiplication of communities of cells which are confined to a restricted volume of nutrient medium,

is an autocatalyzed process (20). That is, up to a certain point, the occurrence of multiplication facilitates further multiplication. Beyond this point either the exhaustion of foodstuffs or the accumulation of the products of growth slows the process and ultimately brings it to a close. Since the cessation of multiplication anticipates the exhaustion of the nutrients, the factor which terminates reproduction must be the accumulation of certain products (19).

It has been shown elsewhere (18) that when two Infusoria (*Enchelys* or *Colpidium*) are isolated together into the same small volume of culture medium, the number of individuals produced within a given period is not double, but much more than double the number of individuals which is produced in the same time in a culture of equal volume which was originally inoculated with a single individual. This mutual acceleration (*allelocatalytic effect*) is not due to conjugation because, under the conditions which prevail in these experiments, conjugation does not occur, and if it had occurred then, as Jennings has shown (4), the effect would have been the reverse of that observed. Evidently some substance issues from the cells which has the effect of accelerating multiplication. When but one individual is initially present the concentration of this substance is small and the rate of reproduction low. When two individuals are initially present the concentration of this substance is higher and the rate of multiplication is, in consequence, enhanced. The process of multiplication is, for this reason, autocatalyzed. The ultimate cessation of reproduction in old, that is, thickly populated cultures, is attributable also to the accumulation of a product of growth, and possibly the same product that was initially responsible for acceleration. This is shown by the fact that mere dilution of the culture with distilled water renders reproduction possible once more, and further by the fact that traces of old culture liquid greatly accelerate reproduction in young cultures.

When individuals are isolated from old cultures into fresh culture fluid, reproduction does not immediately ensue. A period of "lag" elapses before multiplication begins, and the duration of lag is longer the greater the age (or population) of the parent culture (17, 19). The period of lag is not in the slightest degree affected by the presence of other individuals in the culture. Only after the first division is the accelerative effect of the second individual, or of traces of old culture liquid, rendered manifest. We infer that during interdivisional periods the accelerative substance does not issue from or penetrate into the cell. Since the nuclear membrane is dissolved at the time that division occurs, it appears probable that the source of the accelerative substance, and the seat of its action as well, is the nucleus. If this be the case, however, then the accumulation of this substance in the culture medium as the organisms successively divide must result in its more incomplete issuance from the nucleus at each successive division, so that it must accumulate within the nucleus, parallel with its accumulation in the medium. The nuclei of the infusoria which have

been produced within a thickly inhabited culture medium, in which frequent divisions have occurred, therefore, are loaded with the products of previous divisions, and to this is attributable their inability to multiply further. To this, also, we must attribute the phenomenon of "lag" when these infusoria are transferred to fresh culture medium, since the nuclear membrane prevents, or very greatly delays, the issuance of these products from the nucleus. If, therefore, the nuclear membrane could by any means be rendered sufficiently permeable to permit the issuance of these accumulated products the duration of lag would presumably be curtailed. In the duration of the lag period we have, therefore, a possible criterion of the permeability of the nuclear membrane.

Those agents which increase the permeability of the exterior surfaces of cells generally, in the higher concentrations, bring about cytolysis. Among cytolytic agents of this type may be included alternating electric currents, heat, distilled water, saponins, bile salts, and other agents which reduce surface-tension, and lipoid-solvents, such as benzene, chloroform, and alcohols. The following experiments were undertaken with a view to ascertaining the effect of these cytolytic agents upon the duration of lag, and therefore, it is inferred, upon the permeability of the nuclear membrane.

EXPERIMENTAL.

The duration of lag is unaffected by density of population. Whether one infusorian *(Enchelys)* or fifty are introduced into about 50 milligrammes of fresh culture-medium, the period which elapses before the first division occurs is, within very small limits of variation, the same. The products which accumulate in thickly populated culture media, and which are transferred with the infusoria to the new culture, are also without effect upon the duration of lag. The effect of various agencies upon the duration of lag can, therefore, be ascertained by isolating a group of individuals in a very small drop of the parent culture fluid (about 3 milligrammes), counting them, and then adding a large drop (about 50 milligrammes) of fresh culture-medium. The duration of lag is then the time which elapses before the number of individuals in the culture undergoes an increase.

The duration of lag is, however, profoundly affected by the age of the parent-culture (17, 19). It increases with increasing age of the parent-culture up to five or six days, at which time the population of the parent-culture, at temperatures between 15°C. and 20°C. becomes maximal. Thereafter the duration of lag is only slowly affected by further increase in the age of the culture. The individuals inoculated from a seven days' old parent-culture into fresh hay infusion begin to divide, at temperatures between the limits mentioned, in from nineteen to twenty hours. The second division, on the contrary, occurs within four hours after the first.

The hay-infusion used in these experiments was prepared by mixing five grammes of chaffed oaten hay with 100 cc. of distilled water, and heating the mixture to boiling in a 200 cc. capacity Erlenmeyer flask. The flask was transferred to a boiling water bath for one hour, and the extract was then filtered into a steamed flask. The filtrate was transferred to a steamed 100 cc. graduate and the volume made up to 75 cc., if necessary, by the addition of freshly-boiled distilled water. This fluid was then aerated by a stream of air which passed through a bulb packed with sterilized absorbent cotton, and was introduced at the bottom of the column of liquid through a sterilized glass tube connected with the bulb by a freshly steamed length of rubber tubing. After aeration for one hour, 1 cc. of $\frac{N}{10}NaHCO_3$ solution* was added to every 25 cc. of fluid. This mixture constituted the standard culture-medium with which other media were compared. Subcultures of infusoria were always taken from cultures originally inoculated into culture-medium of this composition. The cultures were made upon slides provided with a central depression, which lay upon wet pads of filter paper in covered Petri dishes. The slides were sterilized by boiling before use.

Effect of Distilled Water.

The period of lag is very decidedly abbreviated by inoculation into distilled water previously buffered by the addition of 1 cc. of $\frac{N}{10}$ $NaHCO_3$ solution to every 25 cc. of water. On comparing with parallel cultures in hay infusion, inoculated from the same parent-culture, it is invariably found that the first division, in the distilled-water cultures, is completed before any individuals have begun to divide in the hay-infusion cultures. Since this divison, as remarked above, is speedily followed by a second division, the population of the distilled-water cultures rapidly establishes a lead over that of the hay-infusion cultures. Within forty-eight hours, however, the population of the distilled-water cultures has attained its maximum density (from 3,000 to 4,000 per cc.), while the population of the hay-infusion cultures continues to increase until the fourth or fifth day, when it attains a density of over 60,000 per cc.†

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* The $NaHCO_3$ solution was made up by dissolving the calculated amount of the salt in distilled water and then saturating this solution with CO_2.

† Estimated by filling a white-cell counting pipette to the 0·5 mark (this amount was weighed and found to be ten milligrammes). Distilled water was then drawn in up to the top of the bulb, giving a dilution of one in twenty-two. This mixture was discharged upon a slide and the infusoria counted in one twenty-second of its volume (0·01 cc.). Duplicate estimates agreed within ten per cent.

This effect might conceivably be attributable to either of the following causes. It might be due to starvation, since the foodstuffs contained in such a culture must be practically limited to those introduced with the organisms inoculated into it. Then the P_H of distilled water buffered with $NaHCO_3$ might not be identical with that of hay infusion similarly buffered, if the hay infusion chanced to contain substances capable of reacting with bicarbonates. Finally, it might be due to hypotonicity of the medium. That is really due to this latter factor is shown by the fact that dilution of standard hay infusion, with an equal volume of distilled water, is sufficient to cause appreciable abbreviation of the lag-period. Evidently starvation cannot account for the short duration of lag in such a mixture. That it is not due to excess of bicarbonate unneutralized by substances present in hay infusion, is shown by the fact that the abbreviation of lag due to dilution occurs even if the distilled water employed to dilute the hay infusion is not previously buffered, so that the change of P_H, if any, would be precisely opposite to that involved in exchanging buffered hay infusion for buffered distilled water. The following results illustrate these facts:

Age of parent-culture, seven days.

Culture Fluid.	No. of Individuals initially present.	Individuals present after 19 hours.		Individuals present after 24 hours.	
		Actual number.	Per cent. of initial number.	Actual number.	Per cent. of initial number.
Hay infusion	25	24	96	40	160
One part hay infusion to one part distilled water	39	78	200	157	400
Three parts hay infusion to one part distilled water	26	34	130	78	300
Seven parts hay infusion to one part distilled water	15	16	106	40	266

These results immediately suggested a possible source of fallacy in the estimation of the duration of lag in individuals isolated from old parent-cultures into a fresh culture-medium. Infusoria readily withstand transference from one medium to another which is hypotonic to the first, but they are definitely injured by transference to a medium which is relatively hypertonic. The injury is manifested by excitation of movement, followed by sluggishness, shrinkage in size, and, frequently, death of individuals so transferred. Now, in a densely inhabited culture the foodstuffs originally present in the culture-medium have been converted to a large extent into the protoplasm of Infusoria and accompanying bacteria. These originally contributed to raise the osmotic pressure of the

medium, and their synthesis into protoplasm must result in rendering densely populated culture-media hypotonic in comparison with fresh culture-medium.* Transference of individuals from densely inhabited cultures to fresh culture-medium, which has not been diluted, therefore involves transference of the infusoria to a medium which is hypertonic in comparison with that to which they have become habituated. The possibility suggested itself that the duration of lag might be lengthened by the process of adaptation to a relatively hypertonic medium. Indeed, it appeared to be not impossible that the entire excess of duration of lag over the duration of the succeeding interdivisional period might be attributable to this factor. Accordingly the following experiment was undertaken in order to ascertain whether a process of gradual acclimatization to the new culture-medium would abbreviate the duration of lag. When the old and densely inhabited culture-medium is diluted with an equal volume of fresh culture-medium the change of tonicity is only one-half that which results from transferring the infusoria directly to fresh infusion. Correspondingly, we find that such dilution of the old culture induces but slight excitation of movement and no shrinkage of the cells. If, after residence for an hour in this mixture, infusoria are now transferred to fresh culture-medium the excitation is again relatively slight and no shrinkage of the cells, and few, if any, deaths occur.

A seven days' old culture, densely inhabited by infusoria, was diluted with an approximately equal volume of fresh hay infusion, and from this mixture small samples (about 0·003 cc.) were withdrawn from time to time and placed in the depression on a slide, the individuals contained in the sample were counted, and 0·05 cc. of fresh hay infusion was then added. The following were the results obtained:

Period of Acclimatization.	No. of Individuals initially present.	Number of Individuals after 20 hours.		Number of Individuals after 24 hours.	
		Actual number.	Per cent. of initial number.	Actual number.	Per cent. of initial number.
2 minutes	38	38	100	46	121
10 „	31	30	97	40	129
20 „	7	7	100	8	114
30 „	61	71	116	100	164
45 „	25	35	140	51	204
60 „	24	27	113	44	183
120 „	32	42	131	61	191

* The change of tonicity cannot be large for, as shown above, even dilution to seven-eighths of the usual concentration of hay infusion results in a very slight abbreviation of lag, and dilution to three-fourths of its usual concentration results in definite abbreviation of lag. I have determined the freezing-point depression of freshly prepared hay infusion. It lies between 0·15° C. and 0·17° C., corresponding to a concentration between one-tenth and one-twelfth molecular. One-eighth of this depression is 0·02° C., which is within the limits of error of the estimation. The freezing point of a fairly densely inhabited but not maximally populated culture did not differ by more than this from the freezing point of fresh infusion.

It is evident from these figures that periods of acclimatization exceeding twenty minutes do actually abbreviate the duration of lag to a slight degree. Thus in the cultures inoculated with organisms which had been acclimatized for 30 minutes or more, divisions had begun in 20 hours, and in those which had been acclimatized for 45 minutes or more all, or nearly all, the individuals inoculated had divided in 24 hours. On the contrary, in those cultures which had been inoculated with individuals which had been acclimatized for periods less than 30 minutes, division had not begun in 20 hours, while in 24 hours only about 20 per cent. of individuals had yet divided. Notwithstanding this fact, however, the small abbreviation of lag accomplishable by means of acclimatization indicates very clearly that the greater part of the duration of lag is due to factors other than change of environment, in other words, to the physiological condition of the cells inoculated into the fresh medium. Consequently no great error is introduced into the estimation of lag if acclimatization is omitted, and none at all into the difference of lag periods in different media, provided that in experiments designed to ascertain the effect of modification of the culture medium upon the duration of lag, the controls inoculated into the normal medium and the organisms inoculated into the modified medium are similarly acclimatized, or not acclimatized, as the case may be. In the experiments described hereafter acclimatized individuals were employed in the experiments upon the effects of alternating currents, benzene, chloroform, and alcohol, while in the experiments upon the effects of bile salts and saponin acclimatization was not practised. It is probable, however, that lack of acclimatization is responsible for the deaths which occur in a small proportion of individuals isolated directly into fresh culture media. The occurrence of these deaths has been noted by Cutler and Crump (2), and the experimental results recounted in this paper afford additional illustration of this phenomenon. I have observed that the frequency of deaths is much diminished by acclimatization, while in one instance, in which the filtrate from the heated mixture of hay and water was not made up to 75 cc. with distilled water as usual, but simply aerated and buffered, so that the medium was slightly more concentrated than usual, the percentage of deaths among individuals inoculated into it was exceptionally high.

Effect of Alternating Electric Currents.

It was found that infusoria *(Enchelys),* suspended in hay infusion between platinum wire electrodes 4 mm. apart, are killed within a few seconds by the current from a Harvard Inductorium, in which the primary is excited by one dry cell and the secondary is 3 cm. from the primary. At 6·5 cm. distance of the secondary from the primary the organisms appear to have great difficulty in swimming between the electrodes, and hence tend to congregate close to the electrodes. At 7·5 cm. distance the swimming movements are somewhat abnormal,

but the organisms appear to be uninjured, and continue to swim between the electrodes. At this distance, the front of the secondary coil, in the Inductorium employed, is just exactly level with the rear extremity of the primary coil.

A seven-day culture was diluted with an equal volume of fresh culture medium, and after an hour a small drop of this mixture was placed between platinum points upon a slide and elongated with the point of the pipette until contact was established with both electrodes, which were 4 mm. apart. Current was then passed for varying periods, the electrodes removed, and the individuals in the drop counted. A large drop (0·05 cc.) of fresh culture medium was then added. This was repeated with various durations of exposure to the current and two different strengths of current. The following were the results obtained:

Distance of secondary from primary.	Duration of current.	No. of individuals initially present.	No. of individuals after 20 hours.		No. of individuals after 24 hours.	
			Actual number.	Per cent. of initial number.	Actual number.	Per cent. of initial number.
——	None	32	42	130	61	·191
7·5 cm.	5 mins.	32	48	150	64	200
7·5 cm.	10 mins.	26	25	96	42	158
6·5 cm.	5 mins.	30	36	120	55	183

There is no evidence in these figures of any appreciable abbreviation of lag, and, in fact, the longest exposure to the current seems to have definitely prolonged the period of lag, or, if not that, then individuals which had died subsequently to the passage of the current had only just been replaced by the divisions which occurred prior to twenty hours.

The experiment was repeated on two or three occasions, and no evidence of abbreviation of lag could be obtained. One would infer from this that the permeability of the nucleus is not sufficiently enhanced by currents of this density to permit escape of the nuclear autocatalyst. Of course, this does not necessarily conflict with Lillie's observations upon frog-leucocytes, cited in the introductory part of this paper, because, in the first place, he was able to use higher current densities without destruction of the cells which he employed, and, in the second place, an enhancement of permeability sufficient to permit issuance of one substance from the nucleus might not be sufficient to permit issuance of another.

Effect of Bile Salts.

The mixed bile salts were prepared from ox-bile, purified with charcoal and recrystallized. Varying proportions of this substance were added to hay infusion, and the effects noted upon the duration of lag and the subsequent rate of multiplication. It was found that these infusoria are very resistant to the cytolytic action of bile salts. They are killed within a few minutes by exposure

to a 0·05 per cent. solution, but withstand a solution of one-half this concentration indefinitely. Colloids frequently protect cells from the entrance of water caused by cytolytic agents (15), but the colloids present in hay infusion, at all events, have no appreciable protective effect, since the lethal concentration of bile salts is approximately the same whether the salts are dissolved in distilled water or in hay infusion. The following data illustrate the results obtained:

The parent culture was seven days old.

Culture Medium.	Per cent. of Bile Salts.	No. of Individuals initially present.	No. of Individuals present After 20 hours.	No. of Individuals present After 44 hours.
Hay infusion plus 1 in 25 of				
$\frac{N}{10}$ $NaHCO_3$	None	31	27	160
„	0·625	28	23	115
„	0·391	34	33	195
„	0·156	57	53	345
„	0·078	23	18	103
„	0·039	29	25	195
Distilled water plus 1 in 25				
of $\frac{N}{10}$ $NaHCO_3$	None	63	80	199
„	0·625	23	34	193
„	0·313	55	64	189
„	0·156	52	52	148
„	0·078	50	54	176
„	0·039	47	66	179

It is evident that in all of the distilled water cultures division had begun before it began in the hay infusion cultures. The presence of bile salts in the distilled water cultures did not diminish, nor did it augment, this abbreviation of lag. None of the concentrations of bile salts employed appreciably affected the duration of lag in hay infusion. At twenty hours no divisions had occurred, and by forty-four hours all of the cultures, irrespective of the content of bile salts, contained from five to six times as many individuals as they had contained at twenty hours. The cultures in hay infusion continued to multiply, but the cultures in distilled water had already, at forty-four hours, attained their maximum population, which was not affected by the presence of the bile salts. In the hay infusions, however, the maximal population, attained after seven days, was noticeably diminished by bile salts, as the following figures show:

Concentration of Bile Salts in Hay infusion.	Population per cc. at the end of seven days.
None	100,800
0·625 per cent.	50,400
0·391 „ „	48,300
0·156 „ „	63,000
0·078 „ „	105,000
0·039 „ „	31,000

It is uncertain to what this diminution of final population is due. It may be only apparent and due to delay of the later stages of multiplication. It may be due to a diminution of bacterial population consequent upon the well known disinfectant action of bile salts. This would lead, presumably, to some modification of the availability of foodstuffs. Between these and other possibilities I have at present no data which would enable a decision to be reached.

Effect of Saponin.

Infusoria *(Enchelys)* are even more remarkably resistant to the cytolytic action of saponin than to the action of bile salts. Even a 1 per cent. solution of Merck's saponin is not toxic. When it is recalled that 1 part in 64,000 of saponin will cytolyze 30 per cent. of Echinoderm eggs within an hour (15), the difference of the resistance of these two types of cells to the action of cytolytic agents of this class will be appreciated.

Groups of individuals were transferred directly from seven day old cultures into hay infusion containing varying proportions of saponin. The following were the results obtained:

Parent Culture.	Concentration of Saponin. Per cent.	No. of individuals initially present.	No. of individuals present after 20 hours.	26 hours.	44 hours.
A209	None	23	15	15	37
„	1·000	17	8	8	25
„	0·500	34	19	22	80
„	0·250	17	17	18	60
A208	None	53	40	—	—
„	None	67	59	79	—
„	0·1000	24	20	—	—
„	0·0500	45	40	—	—
„	0·0250	32	28	—	—
„	0·0125	28	25	—	—
„	0·0100	31	29	38	—
„	0·0050	65	70	122	—
„	0·0025	75	88	146	—
„	0·0013	86	80	121	—
„	0·0006	68	63	71	—
„	0·0003	24	19	44	—

There is no evidence of any important abbreviation of lag in any of these mixtures. Since these cultures were inoculated with individuals which had not been acclimatized to fresh culture medium, a number of deaths occurred in nearly all the cultures, with the result that the number of individuals inhabiting the cultures twenty hours after inoculation was in almost every instance less than the number originally introduced. In every culture prepared from the parent culture A209 the lag period equalled or exceeded twenty-six hours. In those prepared from parent culture A208 its duration lay between twenty and twenty-six hours.

The density of population attained one week after inoculation was somewhat diminished by the highest concentrations of saponin, in comparison with the population of the control which, however, happened in this instance to be exceptionally dense. It was appreciably increased by the lower concentrations, as the following figures show:

Parent Culture.	Concentration of Saponin.	Population per cc. after seven days.
A209	None	231,000
,,	1·000	162,800
,,	0·250	92,000
A208	None	88,000
,,	0·1000	90,000
,,	0·0500	167,200
,,	0·0250	114,400
,,	0·0125	127,600
,,	0·0100	112,200
,,	0·0050	101,200
,,	0·0025	127,600
,,	0·0013	154,000
,,	0·0006	176,000
,,	0·0003	72,600

The effect of saponin upon the final density of population, therefore, stands in contrast to that of bile salts. When bile salts and saponin are both added to hay infusion (0·025 per cent. bile salts and 0·0125 per cent. saponin) the mixture is not toxic, the lag-period is neither abbreviated nor delayed, but the final maximal population is diminished to the same extent as by the bile salts alone. The effect of bile salts upon the final density of population, therefore, predominates over that of saponin.

Effect of Ethyl Alcohol.

It was found that hay infusion containing 10 per cent. of ethyl alcohol by volume is lethal for *Enchelys* within two or three minutes. Five per cent. of

ethyl alcohol per volume is, however, devoid of toxicity. To a seven day parent culture an equal volume of fresh hay infusion was added, and after forty-five minutes small volumes of this mixture were withdrawn and added to 0·05 cc. of hay infusion containing varying percentages of ethyl alcohol. The following were the results obtained:

Per cent. of Alcohol by volume.	No. of individuals initially present.	No. of Individuals present after 18 hours.	24 hours.
5·00	30	34	69
2·50	12	13	24
1·25	29	36	70
None	25	24	40

There is no evidence in these results of any important modification of the duration of lag.

Effects of Chloroform and Benzene.

Distilled water was saturated with chloroform or benzene. These solutions were lethal to infusoria within a few minutes, but dilution with an equal volume of distilled water or hay infusion sufficed to render them non-toxic.

To 1, 3, and 7 cc. respectively of hay infusion were added 1 cc. of the distilled water saturated with chloroform or benzene and groups of infusoria, previously acclimatized to fresh hay infusion, were introduced into 0·05 cc. portions of these mixtures. Controls were inoculated into similar dilutions of hay infusion with distilled water. In every case division commenced at the same time in the media containing chloroform or benzene as in the control media similarly diluted, which did not contain these reagents. Chloroform and benzene, therefore, in non-lethal dosages, are without effect upon the duration of lag.

Effect of Heat.

The effect of heat upon the permeability of the nuclear membrane cannot be directly ascertained by this method, because a rise of temperature might be expected to accelerate the synthesis of nuclear material and other reactions involved in reproduction (12), and therefore, in any case, to abbreviate the duration of lag.

It has previously been shown, however, that a temperature of 30°C. is lethal for isolated infusoria *(Enchelys)*, but that a considerable number of individuals, isolated together into a similar small volume of hay infusion, not only survive exposure to this temperature, but even multiply (17). This result cannot be attributed to the introduction of greater numbers of bacteria together with greater numbers of infusoria into the fresh hay infusion, because the viability of isolated

individuals at 30°C. is not in any way increased by previous growth of bacteria in the infusion. There is evidently a mutual protective influence arising from the contiguity of cells. We have seen that contiguity of cells enables them to multiply more rapidly because less of the nuclear autocatalyst is shed from the nucleus at each division, on account of its higher concentration in the medium. It appears possible that the protective effect of contiguity at high temperatures may originate in the same manner, and, if this be admitted, then we must attribute the death of isolated infusoria at 30°C. to excessive loss of autocatalyst from the nucleus.

Since the death of isolated individuals occurs before division, we must refer this loss to increased permeability of the nuclear membrane.

Unless the protective effect of mutual contiguity at high temperatures has a different origin from the accelerative effect of mutual contiguity upon division-rate at lower temperatures, therefore, we must conclude that the permeability of the nuclear membrane is increased by rise of temperature. Further evidence is requisite to enable us to decide between these alternative possibilities.

From all of these observations it is clear that, with the exception of hypotonicity and the possible exception of rise of temperature, none of those agencies which commonly lead to increase of the permeability of the exterior surface of the cell have any effect upon the permeability of the nuclear membrane.

CONCLUSIONS.

1. Infusoria may be transferred to relatively hypotonic media without injury, but transference to relatively hypertonic media causes shrinkage of the cells, and if the degree of hypertonicity is sufficient it is fatal. Where the injury is insufficient to lead to death, it nevertheless results in a slight prolongation of the period of lag.

2. Since inhabited cultures of infusoria progressively decrease in osmotic pressure the inoculation of these infusoria into new culture medium, of the same composition as that originally employed in forming the parent culture, involves transference of the infusoria to a relatively hypertonic medium. This is the cause of the deaths which occur in subcultures of infusoria during the first twenty-four hours after inoculation.

3. This lethal result of inoculation into fresh culture medium may be avoided by a process of acclimatization, consisting of dilution of the parent culture with an equal volume of fresh culture medium one hour before withdrawal of the individuals which are to form the subcultures.

4. The duration of lag is not affected in any measurable degree by non-lethal doses of any of the following cytolytic agents: Alternating electric current, bile salts, saponin, alcohol, chloroform, and benzene.

5. It is inferred that the conditions which determine the permeability of the

nuclear membrane are totally different from those which determine the permeability of the exterior surface of the cell.

6. Inoculation of infusoria into a decidedly hypotonic medium (distilled water containing 4 cc. per cent. of $\frac{N}{10}NaHCO_3$) results in a very considerable abbreviation of the lag period. It is inferred that this procedure leads to an increased permeability of the nuclear membrane.

7. From the effect of density of population in protecting infusoria from the lethal effects of rise of temperature, it is inferred that rise of temperature may possibly also increase the permeability of the nuclear membrane.

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STUDIES ON INSULIN, I.
PREPARATION AND STANDARDIZATION OF INSULIN.

by

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The standardization of insulin for clinical use has become a matter of importance, both clinically and commercially. The method at present in use, namely, the estimation of the fall in blood sugar in rabbits after administration of insulin, entails considerable labour and expense, as the results are often inconclusive, necessitating the use of a large number of animals. As mice are more economically reared, more quickly and easily handled, and, under constant conditions, give very consistent results, it may prove an advantage to use them in the assay of insulin.

The following experiments were carried out with a view to determining the value of a "mouse-unit" of insulin in terms of the two-kilogram rabbit unit.

The insulin used in these experiments was prepared from ox pancreas by a modification of the method of Robertson and Anderson (4).

Method of Preparation.

The pancreas, on being collected at the slaughter-house, is immediately placed in shallow trays in the freezing chamber (temperature—15°C.) and frozen into solid blocks. In this condition it can be kept, if necessary, for several weeks without appreciable loss in the subsequent yield of insulin. The pancreas is minced while still frozen, and placed in an equivalent volume of methylated alcohol, acidified by the addition of 30 cc. concentrated hydrochloric acid (36 per cent.) to the litre of alcohol. A typical batch consists of about twelve kilograms of minced tissue. A small amount (100 cc.) of ether is added to assist the cytolysis of the cells, and the whole thoroughly stirred for several hours. The mixture is then put into a filter press and the liquid squeezed out. To every four litres of the extract is added one kilogram of exsiccated sodium sulphate.

The sodium sulphate combines with water to form Na_2SO_4 $10H_2O$, thus increasing the concentration of the alcohol and causing the coagulation of the proteins present in the mixture. These, together with the sodium sulphate, can be removed by means of the filter press, and finally by filtering through paper. The clear filtrate is now placed in shallow pans in a serum dryer (manufactured by Chas. Hearson & Co., Ltd., London), and reduced to about one-tenth its volume by means of a current of warm air (40°C.). The thick viscid mass is then repeatedly shaken up with ether in a separatory funnel until the fat is removed. The surplus ether is evaporated in the serum dryer. This is done to prevent the precipitation of insulin in the next step. When no further smell of ether can be detected, enough absolute alcohol is added to bring the alcohol content of the whole to 80 per cent. This will precipitate salts and protein residues. After standing for an hour, the precipitate is filtered off through paper, and the P_H of the filtrate is adjusted by the addition of 10 per cent. caustic soda to about the iso-electric point of insulin (P_H 5·0). In a typical twelve kilogram batch this filtrate measures about one litre. The insulin is precipitated by pouring this filtrate in a thin stream into eight times its own volume of a mixture of equal parts of absolute alcohol and ether (4 litres absolute alcohol and 4 litres ether). The insulin precipitates in the characteristic dense flocculent cloud, which slowly sinks to the bottom of the vessel. After standing overnight in the cold room (5°C.), as much as possible of the clear supernatant liquid is poured off and the remainder centrifuged. The precipitate dissolves readily in water or ·85 per cent. sodium chloride solution, if the hydrogen ion concentration is adjusted to P_H2. Any remaining ether can be driven off by subjecting the solution to a reduced air pressure. At P_H2 the insulin made by this method will keep for at least six months at ordinary room temperatures, and without apparent loss of potency.

The yield from a typical twelve kilogram batch is about 3,000 two-kilogram rabbit units, or 9,000 clinical units; that is, 1 clinical unit per 1·3 gm. of minced tissue. An aliquot part of the final aqueous solution is taken for standardization.

Method of Assay.

In using mice for the assay of insulin it is necessary to keep certain conditions as constant as possible. The mice used in the following experiments were all fed on maize and rice boiled in fresh milk, with hard-boiled egg once a week. They were all starved for twenty-four hours before being used for standardization, and none of them weighed less than 24, or more than 26 gms. Before being used they were also examined for sores or any condition tending to disturb the normal body temperature. They are kept normally in a room, the temperature of which is regulated to about 15°C., but after the administration of insulin they were kept at a constant temperature of 33°C., and it was found that any variation in this factor, such as removal for five minutes to normal room temperature (15°C.)

often caused a wide variation in results obtained. For example, an amount of insulin sufficient to cause convulsions in a mouse kept at a room temperature of 33°C. would not bring about such symptoms in one kept at a room temperature of 15°C., and frequently the convulsions in the first case would subside on the animal being removed to a cooler room. A. Krogh (cited by Macleod) (3) observed that mice after administration of insulin rarely display convulsions at ordinary atmospheric temperatures. He noticed that the most constant symptom was a fall in the body temperature, accompanied by general weakness. It is evident, therefore, that any method of assay entailing the use of the convulsive symptoms must include this condition of temperature. When the factors of diet, weight, and temperature are strictly controlled, however, the results obtained are constant to a very high degree.

In standardizing concentrated solutions of insulin on mice it is necessary to use very high dilutions (e.g., 1 in 500, or 1 in 1,000). The solutions should be made with slightly acidified water, and should be used immediately, as standing even for half an hour may bring about a decrease in potency. The doses were injected under the skin at the back of the neck, as it was found that in this position there is less chance of leak from the puncture than in the skin of the back or belly. A primary assay is conducted on ten or twenty mice to determine the approximate convulsion level. For example, as follows:

TABLE I.

Weight, 24-26 grams. Starved twenty-four hours. Kept at room temperature 33° C. after subcutaneous injection. Dilution, 1 to 500.

Mouse.	Dose.	Result.	Remarks.
1	1 cc.	Convulsions 30 minutes.	
2	·9 cc.	No convulsions.	No. 2 is obviously an erratic. Such a mouse is excluded from further assays.
3	·8 cc.	Convulsions 20 minutes.	
4	·7 cc.	„ 20 „	
5	·6 cc.	„ 30 „	
6	·5 cc.	„ 35 „	
7	·4 cc.	„ 35 „	
8	·3 cc.	„ 35 „	
9	·2 cc.	No convulsions.	
10	·1 cc.	No convulsions.	

TABLE II.

Conditions of diet, weight, and temperature as in Table I. Dilution 1 to 1,000.

Mouse.	Dose.	Result.	Remarks.
1	1 cc.	Convulsions 20 minutes.	
2	·9 cc.	„ 20 „	
3	·8 cc.	„ 35 „	
4	·7 cc.	„ 20 „	
5	·6 cc.	„ 40 „	
6	·5 cc.	Weak.	As in No. 6, general body weakness without convulsions may occur. It is noted in the approximate assay, but discarded in the more accurate determinations.
7	·45 cc.	Convulsions 25 minutes.	
8	·4 cc.	No convulsions.	All mice in convulsions recovered on a small subcutaneous injection of glucose.
9	·35 cc.	„ „	
10	·3 cc.	„ „	
11	·2 cc.	„ „	
12	·1 cc.	„ „	

It is now obvious that the convulsion level lies somewhere between ·4 cc. and ·5 cc. of a dilution of 1 to 1,000.

Fifteen mice are now taken and divided into three groups of five. The animals of Group A receive each a dose of ·5 cc., those of Group B one of ·45 cc., and those of Group C ·4 cc. Of the five mice in Group A all convulsed. Of Group B three convulsed. Of Group C one convulsed. The procedure is repeated, using ten mice, and dosing five with ·45 cc. each (Group D), and five with ·4 cc. each (Group E). Four of Group D convulsed and one of Group E. ·45 cc. of a dilution of 1 to 1,000 is, therefore, taken as the "mouse unit," and can be confirmed, if necessary, by using higher dilutions.

In order to determine the relation of the mouse-unit to the two kilogram rabbit-unit, 1 cc. of the above sample of insulin was diluted to 10 cc. and used for assay. The rabbits were albinos, and had been fed on grass and wheat. For two days previous to the experiment they were fed on grass only, and for the twenty-four hours immediately prior to the assay they were deprived of food, but given water.

TABLE III.

Rabbit.	Body Weight. Kg.	Dose per kg. body weight.	Symptoms.	Blood Sugar (McLean's Method).
1	2	1 cc.	Convulsions 1¼ hrs.	·03% at 1½ hrs. after injection.
2	1·9	·8 cc.	„ 1¼ hrs.	·03% at 1½ „ „
3	1·7	·7 cc.	„ 1½ hrs.	·05% at 1½ „ „
4	1·9	·6 cc.	„ 1½ hrs.	·06% at 1½ „ „
5	2	·5 cc.	„ 1½ hrs.	·04% at 1½ „ „
				·03% at 1¾ „ „
6	1·7	·4 cc.	No convulsions	·08% at 2½ „ „
7	1·8	·3 cc.	Convulsions 3 hrs.	·05% at 1½ „ „
8	1·6	·2 cc.	No convulsions	·10% at 2 „ „

Taking ·45 cc. of a dilution of 1 to 1000 as the mouse-unit, and, say, ·35 of a dilution of 1 to 10 as the rabbit-unit, we might take the rabbit-unit as approximating roughly 160 mouse-units. A more accurate assay on a large number of animals was now carried out to determine this. A sample of insulin hydrochloride (made by the method of Dudley) (1) was dissolved in distilled water at $P_H 2$, and used for the experiment. Thirty-seven rabbits were used and eighty-five mice. Of a dilution of 1 to 10, ·5 cc. per kilo body weight caused convulsions with a drop in the blood sugar to below ·05 per cent., while ·4 cc. per kilo produced no convulsions, nor were we able to find a decrease in the blood sugar. The mice, as before, were dosed in groups of five. One cubic centimetre of the hydrochloride solution was diluted to 250 cc. with acidified distilled water. The mice were divided into Groups A, B, C, D, E, and dosed as follows:

TABLE IV.

Dil. 1 : 250. Av. Weight 26 gms. Room Temperature 33°C.

Group.	Dose.	Result.	Remarks.
A. 5 mice	1 cc.	All convulsed.	
B. „ „	·8 cc.	„ „	
C. „ „	·6 cc.	„ „	
D. „ „	·4 cc.	„ „	
E. „ „	·2 cc.	Four convulsed.	Group E may indicate the nearing of the minimum convulsion dosage.

A further twenty-five mice were taken and dosed as follows:

TABLE V.

Dil. 1 : 250. Av. Weight 26 gms. Room Temperature 33°C.

Group.	Dose.	Result.	Remarks.
A. 5 mice	·5 cc.	All convulsed.	
B. „ „	·4 cc.	Four convulsed.	
C. „ „	·3 cc.	Three convulsed.	The "unit" lies evidently
D. „ „	·2 cc.	Two convulsed.	between ·1 cc. and ·3 cc.
E. „ „	·1 cc.	One convulsed.	of this dilution.

Fifteen mice are now divided into three groups and dosed to confirm these findings.

TABLE VI.

Dil. : 150. Av. Weight 26 gms. Room Temperature 33°C.

Group.	Dose.	Result.	Remarks.
A. 5 mice	·3 cc.	Four convulsed.	·3 cc. of this dilution
B. „ „	·2 cc.	One convulsed.	is taken as the "mouse-
C. „ „	·1 cc.	None convulsed.	unit."

These findings were now confirmed by using 1 cc. of the hydrochloric solution diluted to a litre. ·6 cc. of this dilution caused convulsions in three out of five mice, ·5 cc. caused no convulsions, and ·7 cc. produced convulsions in five out of five mice. ·6 cc. was, therefore, taken as the unit. We calculated on these figures that the original hydrochloride solution contained $\frac{5,000}{3}$ mouse-units per cc. and 10 two-kilogram rabbit-units per cc. This would indicate that the two-kilogram rabbit-unit contained approximately 167 mouse-units; a somewhat higher value than that obtained by the first assay. The second assay, however, was carried out with greater care and on a larger number of animals. It was, moreover, repeated two weeks later on the same animals with practically identical results.

DISCUSSION.

Attempts to establish a "mouse-unit" of insulin have led to very diverse results. Krogh, cited by J. J. R. Macleod and M. D. Orr (3) has found "that the mouse compared on a body-weight basis is about five times more sensitive to insulin than the rabbit." He defines the mouse-unit as the amount which will cause "definite symptoms" in 50 per cent. of the injected animals, under constant conditions of diet and temperature. It is in the definition of symptoms that confusion may arise. Mice dosed with large quantities of insulin frequently

become comatose, with no evidence of convulsions. On the injection of small quantities of glucose convulsions manifest themselves before the animal finally recovers. Again, when the injection of insulin is nearing the minimum amount that will cause convulsions, the animal may become weak, crawling about the cage with dragging limbs, or lying motionless on one side, yet displaying no convulsive movements. In these experiments convulsions only were taken as the "definite symptoms," and any group of animals showing ambiguous results was discarded, a fresh group chosen, and the assay repeated.

Frazer (2), of Toronto, also found very high values for his mouse-rabbit unit determinations. But we note that the average weight of his mice was 18 grams, and that the insulin used was injected intraperitoneally. It may prove that mice are more sensitive to insulin injected in this manner. This point is being investigated. As the incidence of convulsions is considered by several authors to depend on the *rate* of fall in the blood sugar, the rapidity with which insulin is absorbed would certainly play an important rôle in the production of these symptoms. The discrepancy between the reported ratios of the mouse and rabbit units, and those of Frazer, may be explained by the difference in the rates of absorption of the insulin in the two sets of experiments. The experience of the author tends to show that subcutaneous injections give remarkably constant results, and the method was adopted for this reason. Moreover, such a procedure is more comparable to that employed in rabbit standardization. The subcutaneous method has a further advantage in that the solutions and instruments used need not necessarily be sterilized, as infection is extremely rare under these conditions.

SUMMARY.

An attempt has been made to establish a mouse-unit of insulin in terms of the two-kilogram rabbit-unit.

The "mouse-unit" may be defined as the minimum amount of insulin injected subcutaneously necessary to cause definite convulsions in 60 per cent. of the injected animals, under constant conditions of diet, body-weight, and room temperature.

Under the given conditions, about 167 mouse-units are equivalent to one two-kilogram rabbit-unit. The method used gives very constant results, and is advantageous in that large numbers of animals can be reared and handled cheaply and quickly.

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STUDIES ON INSULIN, II.
THE EFFECT OF HYDROGEN-ION CONCENTRATION ON THE ACTIVITY OF INSULIN SUBJECTED TO HIGH TEMPERATURES

by

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A solution of insulin was divided into three portions, A, B, and C, of equal volume. The hydrogen ion concentration of specimens A and B was adjusted to P_H4 and P_H2 respectively. The third specimen, C, was adjusted to P_H2 and left standing in the cold room at 6°C.

Specimens A and B were autoclaved at 125°C. for half an hour, the steam pressure being about 20 lb. On the following day all three specimens were standardized on white mice according to the method cited in the previous paper.

Specimen A, P_H4, *autoclaved.*

1 cc. of a dilution of 1 : 500 caused convulsions in a 26 gm. mouse thirty minutes after sucutaneous administration.

Specimen B, P_H2, *autoclaved.*

·3 cc. of a dilution of 1 : 1000 caused convulsions in a 26 gm. mouse thirty minutes after subcutaneous administration.

Specimen C, P_H2, *not autoclaved.*

·1 cc. of a dilution of 1 : 1000 caused convulsions forty minutes after administration.

It follows from these figures that specimen A contained 500 mouse-units to the cc., specimen B 3333·3 mouse-units per cc., and specimen C 10,000 mouse-units per cc. This shows a loss of 95 per cent. activity for insulin autoclaved at P_H4, and about 66 per cent. loss at P_H2.

These specimens of insulin were clear and free from precipitates before autoclaving. After subjection to the high temperature a white granular precipitate appeared, and the standardization sample was taken after the samples were thoroughly shaken to ensure an even distribution of the particles through the liquid. In order to find out which contained the active principle, the

precipitate or the filtrate, the specimens were centrifuged; 1 cc. of the clear liquid diluted to 25 cc. with distilled water was used for standardization. Of four mice, dosed respectively with 1 cc., ·75 cc., ·5 cc., and ·25 cc. of this liquid, none convulsed.

The supernatant liquid, measuring 72 cc., was now poured off, an equal volume (72 cc.) of distilled water was added to the precipitate, and the mixture shaken. On the P_H being adjusted to neutrality the precipitate dissolved. 1 cc. of this solution was diluted to 25 cc. and used for standardization. Of five mice, dosed respectively with ·5 cc., ·4 cc., ·3 cc., ·2 cc., ·1 cc., all convulsed. It is thus evident that the active principle was largely, if not wholly, contained in the precipitate.

An aqueous solution of insulin prepared in the same manner as the former, but from another batch of pancreas, was divided into specimens A and B, each measuring 100 cc., and both were adjusted to P_H2. Specimen A was autoclaved for twenty minutes at 125°C., while specimen B was kept at 6°C. 1 cc. of A was diluted to 250 cc. with distilled water and used for assay; ·9 cc. caused convulsions in mice within forty-five minutes.

1 cc. of B was similarly diluted, and ·7 cc. of this solution caused convulsions in mice within the same period of time.

The first specimen, A, contained approximately 278 mouse-units to the cubic centimetre, while the control specimen, B, contained 357 mouse-units per cc.

In this case, the loss due to autoclaving only amounted to about 22 per cent. The diminished loss in this, as compared with the former experiment, is evidently correlated with the non-appearance, in this instance, of the precipitate which, in the former experiment, carried down the active principle with it. No reason can be assigned why a precipitate appeared in the one case and not in the other, but presumably some unnoticed difference in the methods of preparation employed in the two batches led in the earlier experiment to the presence of an impurity, which became insoluble at 125°.

It will be noticed that in the first experiment in which a precipitate formed at 125°C. the solution of insulin was very concentrated, containing 10·000 mouse-units per cubic centimetre. It was thought that possibly this may have been the reason for the formation of the precipitate. We were unable to obtain a further supply of such active material, but two specimens were prepared at P_H2, one containing about 2,000 and the other about 600 mouse-units per cc. On autoclaving at 125°C. for twenty minutes, a precipitate formed in both these samples.

SUMMARY.

It is evident that, provided no precipitate is formed, insulin solutions at P_H2 can be autoclaved at a temperature of 125°C. for twenty minutes, with only between 20 and 25 per cent. loss of activity, while at P_H4 their activity is almost wholly destroyed by this procedure.